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Practitioner's Docket No. 700157-48012

**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of: David E. Fisher

Application No.: 09/229,283

Group No.: 1642

Filed: 1/13/1999

Examiner: UNGAR, Susan

For: USE OF MICROPHTHALMIA FOR DIAGNOSIS, PROGNOSIS AND/OR  
TREATMENT OF MELANOMA

Mail Stop AF  
Commissioner for Patents  
P. O. Box 1450  
Alexandria, VA 22313-1450

**EXPEDITED  
RESPONSE UNDER  
37 C.F.R. 1.116**

**SUPPLEMENTAL AMENDMENT UNDER 37 C.F.R. 1.116**

Dear Sir:

Supplemental to the Amendment Under 37 C.F.R. 1.116 filed May 20, 2004, Applicant is enclosing an executed copy of Dr. Fisher's Declaration.

A Petition for Two-Month Extension of Time to be charged to the NIXON PEABODY LLP Deposit Account No. 50-0850 is submitted herewith.

The Remarks begin at page 2.

### REMARKS

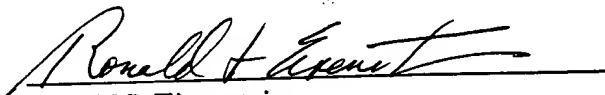
Applicant respectfully submits a copy of the executed Declaration of Dr. Fisher.

In view of the foregoing, the Applicant respectfully submits that all claims are in condition for allowance. Early and favorable action is requested.

Applicant authorizes payment of \$155.00 (the difference between this extension fee and the previously submitted one-month extension fee) to the Nixon Peabody LLP deposit account number 50-0850. The Commissioner is further authorized to charge fee deficiencies or credit overpayments with this submission to said account.

Respectfully submitted,

Date: June 18, 2004



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DECLARATION OF DAVID E. FISHER

I, David E. Fisher, hereby declare as follows:

1. I am Director of the Melanoma Program, Department of Medical Oncology and  
Department of Pediatric Hematology/Oncology at the Dana-Farber Cancer Institute, Inc., Boston,  
MA.

2. I am Associate Professor at the Harvard Medical School, Boston, MA.

3. I am also the inventor of the above-described application.

4. I am familiar with the comments raised by the U.S. Patent Office in an Office

Action mailed August 19, 2003.

5. I disagree with the conclusions made by the Examiner for the following reasons.

6. I taught a method for diagnosing melanoma that involved contacting a biological  
sample containing malignant cells with a probe that recognizes microphthalmia. At the time of  
the patent application, we were abbreviating that name as Mi. Since that time, the protein has  
been called microphthalmia-associated transcription factor abbreviated MiTF or MITF. I taught  
that the skilled artisan, typically a pathologist, could diagnose melanoma by taking a specimen  
containing malignant cells and using probes that bind to Mi where the expression of Mi in a

malignant cell is indicative of melanoma. Thus, the claim specifically teaches the skilled artisan not to just look for the expression of MiTF, but for the expression of MiTF in a malignant cell. Although not explicitly stated people in the field understand that when using a probe they cannot just use it in isolation. They have to use it with what their training has brought to the field. As we teach in our application, the probe can be an antibody (immunohistochemistry) or the probe can be a probe that detects the present of mRNA.

7. The use of antibodies is one of the most important methods of providing malignancy diagnostics. That value of immunohistochemistry is recognized in Chapter 13 of the textbook Cancer: Principles in Practice of Oncology, Fourth Edition, edited by DeVita, V., Hellman, S. and Rosenberg, S. (copyright 1993), wherein the text states at page 231:

The immunohistochemical method has contributed more than any other special technique to the histopathologic diagnosis of tumors.

The chapter goes on to state that the specificity of antibodies adds very useful information to the collective body of data which a pathologist employs in arriving at a diagnosis. Namely, the skilled artisan does not look at a single point in isolation. For example, in being directed to determine expression of Mi in a malignant cell, the skilled artisan would know that they must use other available criteria in looking at a specimen to identify the malignant cell expressing MiTF (Mi).

8. The Examiner stated that osteoclasts, mast cells and melanocytes, which also express MiTF, might also be present in samples. This would not cause any problem because the skilled artisan would know that osteoclasts are multinucleated whereas melanoma cells have only one nucleus; mast cells are filled with basophilic granules whereas melanoma are not; and melanocytes are not invasive cells whereas melanoma cells are. Thus, by looking at the specimen, the skilled artisan would be able to distinguish which cells express Mi in malignant cells and not

be bothered by the potential presence of osteoclasts, mast cells and melanocytes. Moreover, osteoclasts and mast cells are among the rarest cells in the body so the likelihood of their appearing in a specimen is extremely low. Nevertheless, there would be no ambiguity between osteoclasts, mast cells and melanocytes as opposed to a melanoma cell to the skilled artisan, i.e., a pathologist. It should be remembered that in pathology when one is talking about diagnosis what one is doing is narrowing the list of things that could potentially satisfy the diagnosis. One is not necessarily talking about 100% certainty, and the skilled artisan would recognize that because we stated that the expression of Mi in a malignant cell is indicative of melanoma.

9. The Examiner also discusses at page 3 of the Office Action that "although a review of Figure 6 [of my application] reveals differential staining of melanoma in situ as compared to normal control, not only malignant cells but also benign nevus and dysplastic nevus cells which are not malignant" show staining. Again, the person skilled in the art would not be confused and would not identify such cells as being malignant. The reason for this is that differentiating melanoma from benign melanocyte lesions requires no antibody. When a specimen is brought out of the operating room, pathologists routinely look at cells to determine whether they are malignant or benign using certain standard stains. We talk about looking for expression of Mi in a malignant cell. The skilled pathologist knows how to differentiate a malignant cell from a non-malignant cell and routinely does so. This is standard practice.

10. My application expressly talks about diagnosing melanoma by stating that the expression of Mi in the malignant cell is indicative of melanoma. The Examiner states that the application does not teach distinguishing melanoma from other tumors. I disagree. First, we are stating that the expression of Mi in a malignant cell is indicative of melanoma. As the artisan knows and as the word "indicative" indicates, I am not talking about absolute 100% certainties.

None of the current markers on the market give absolute certainties. As I will discuss below, I believe the research confirms that this marker is extremely good at distinguishing melanoma from other tumors — better than the markers that were being used at the time. Therefore, the Examiner's statement that two non-melanoma tumors were found to stain positive for Mi would be of no consequence to the skilled artisan.

11. Indeed, I find the Examiner's discussion of the two non-melanoma tumors somewhat out of context. We indicated explicitly at that point that Mi stains cytoplasm in two of 81 cases and in no cases was there nuclear staining. Thus, in only 2 out of 81 non-melanomic malignant cases, in only about 2.5%, was there any staining, and the staining was in the cytoplasm not the nucleus.

12. Further, on page 232 in Chapter 13 of textbook discussed above, several particularly valuable antibody diagnostics are discussed. Included among these examples are S-100 and HMB-45. As stated in the text, both of these standard diagnostic reagents are imperfect in their sensitivity and specificity, yet, nonetheless are extremely valuable for cancer diagnosis. This text was written prior to our invention.

13. In fact, in the present application in Figures 7, 8 and Table 1 and the discussion at pages 25 through 27, a comparison is made between the present method of using an MiTF probe relative to S-100 and HMB-45. As shown, our probe was more sensitive and more specific than either of those other two antibodies which are nonetheless cited as standard and valuable diagnostic reagents in the text cited above.

14. Just as we taught in the application, people have found that the use of a probe for MiTF is extremely useful. See Chang, K.L. and Folpe, A.L., Advances in Anatomic Pathology, 8:273-275 (2001).

15. The Examiner has contended that we have not addressed the ability of an Mi probe to discriminate between the isoforms. We taught that one should use a probe for Mi and that such a probe will work. The fact that certain isoforms may be found in different places does not affect our teaching. Namely, that the presence of Mi in a malignant cell is indicative of melanoma. With respect to specific antibodies, we are discussing probes that selectively recognize the expression of Mi. Given the teaching and the exemplification of the type of results obtained in the examples, one can readily determine if one has obtained the appropriate antibody.

16. With respect to the question of whether or not one can diagnose melanoma with a probe that detects the presence of mRNA expressing Mi in malignant cells, we taught that you could. The Examiner contends that there are examples where protein levels do not correlate with steady state RNA levels or alterations in mRNA levels. However, that was not what we taught. In fact in the majority of cases, protein and RNA expression levels do correlate, and as in the majority of cases, mRNA levels of MiTF and protein expression levels do indeed correlate. This is explicitly confirmed at page 334 left-hand column of Du, J. AJP, 163:333-343 (2003) ("Protein and mRNA levels of the three antigens [MiTF, SILV and MLANA] were found to correlate in a panel of melanoma cell culture lines as well as human primary melanocytes.") Consequently, as we taught, one can use a probe that looks at mRNA expression levels of Mi.

17. Finally, we wish to emphasize that one of the problems that occurs in recognizing melanomas from other malignant cells is that many melanomas lose pigmentation particularly as they progress and metastasize. Thus, these tumors represent significant diagnostic dilemmas. I found and taught that by contacting a biological specimen containing malignant cells with a probe for MiTF the skilled artisan can then determine the presence (or conversely, the absence) of Mi in such malignant cells and the results can be diagnostic for cancer. If it is present in the malignant

cell, it is indicative of the cells origin, and it is a melanoma. We believe that the method we teach can readily be practiced as taught by the skilled artisan.

18. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



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David E. Fisher



## CHAPTER 13

# Principles of Oncologic Pathology

Oncologic pathology is the branch of pathology that deals with the characterization of neoplasms on the basis of morphologic (shape-related) features, whether at the gross, microscopic, or ultrastructural level. It is a powerful tool for the evaluation of the common traits and differences among the innumerable types of neoplasms that can affect the human body, and it remains the basis for their identification and classification. Oncologic pathology provides clues to the genesis of these tumors and allows fairly accurate predictions about their natural history. The discipline is constantly being enriched by new technologies such as immunohistochemistry or *in situ* hybridization, which in most instances complement rather than replace conventional methods.

The morphologic study of human tumors in this century developed along two separate roads that eventually merged. The first emphasized the study of the nature (and, by inference, the histogenesis) of tumor cells and resulted in elaborate classifications based on analogies with the corresponding normal adult and embryonal counterparts; the second concerned itself with the expected behavior of tumors as judged from their appearance and therefore with the concepts of benignancy and malignancy. Current classifications of human neoplasms reflect, in an imperfect and sometimes imprecise manner, the attempt to name and arrange tumors according to a combination of histogenetic and behavioral traits.

### CLASSIFICATION OF TUMORS

#### HISTOGENESIS

The traditional principle of tumor histogenesis, which has dominated the thinking in this field for more than a century,

is that neoplasms characterized by a certain phenotype arise from normal cells of similar phenotype. According to this scheme, rhabdomyosarcoma is viewed as a tumor originated from skeletal muscle cells, synovial sarcoma as a tumor originated from synovial cells, and so forth. As logical as this assumption appears, it is difficult to reconcile with the observations that some tumors show features of two or more distinct cell types and that some neoplasms occur where their presumed normal parent cells are absent (e.g., synovial sarcoma arising at a distance from a synovial membrane, or osteosarcoma developing away from the skeletal system). Considerable evidence has accumulated in recent years to indicate that this histogenetic assumption is incorrect, and that most if not all neoplasms arise from immature cells, which in the course of neoplastic transformation acquire phenotypic features equivalent to those of one or more normal cell types. More often than not, this differentiation develops along lines analogous to those expected under normal conditions for that particular cell. For instance, primitive epithelial cells located at the base of the crypts of Lieberkühn in the intestinal mucosa differentiate under normal conditions along one or another of four specialized cell lines: absorptive cell, goblet cell, enterochromaffin cell, and Paneth cell. Correspondingly, neoplasms arising from these primitive cells do differentiate along one or another of these various pathways, sometimes singly and sometimes in combination (although in substantially different percentages).<sup>1</sup>

This change in interpretation does not mandate a change in terminology. The names assigned to the various tumors remain the same but have acquired a different significance. Therefore, rhabdomyosarcoma is no longer defined as a malignant tumor *arising* from striated muscle cells, but as a malignant tumor *differentiating* in the direction of striated muscle cells.

## BEHAVIOR

The traditional classification of neoplasms by behavior is into benign and malignant types. These designations are determined by the expected behavior of the tumor rather than its microscopic appearance, although the two parameters are closely related. In the many cases in which there is a divergence, the behavioral aspects take precedence as far as terminology is concerned. For example, the better-differentiated chondrosarcomas have a microscopic appearance similar to that of normal cartilage, whereas some parathyroid adenomas are highly atypical and pleomorphic.

The division of tumors into benign and malignant represents a gross oversimplification of the wide behavioral range exhibited by these lesions, in terms of local aggressiveness and metastatic potential. Two interesting semantic changes have taken place recently in this regard. In the past, tumors were often designated malignant if they had the capacity to metastasize but also if they manifested an aggressive behavior locally. The tendency now is to restrict the term *malignant* to tumors with metastasizing properties. Several changes in terminology have occurred because of this policy, particularly in the field of soft tissue tumors. Tumors formerly diagnosed as well-differentiated liposarcomas in the superficial soft tissue are now commonly diagnosed as atypical lipomas.<sup>2</sup> The other development has been the creation of a new tumor category between the benign and malignant types, variously designated (depending on the site) as *borderline*, *intermediate*, or *undetermined*. Most tumors so named represent malignancies of such a low grade that a cure can be achieved in many cases by a conservative therapeutic approach. The best examples are in the field of gynecologic pathology and are represented by borderline serous tumors of the ovary and smooth muscle tumors of undetermined malignant potential of the uterine corpus.<sup>3,4</sup>

## GRADING OF TUMORS

Determination of the microscopic type of a malignancy does not always provide all the information needed to predict the clinical course or to decide on the therapy. For instance, diagnoses such as prostatic adenocarcinoma or liposarcoma span an extremely wide range of lesions, from the slow growing, rarely metastasizing, and highly curable to the rapidly growing, often metastasizing, and rarely curable. Microscopic grading is an attempt to determine the degree of malignancy independently from cell type and is based on the evaluation of several parameters, which vary depending on the system being studied. They include cellularity, pleomorphism, mitotic activity, type of margins, amount of matrix formation, and presence of hemorrhage, necrosis, or inflammation. Not surprisingly, many of these parameters are closely interrelated. For soft tissue tumors, the number of mitotic figures, and extent of necrosis seem to be the most important parameters.<sup>5,6</sup>

The number of grades varies from system to system, but in general the three-grade system (well differentiated, moderately differentiated, and poorly differentiated, or undifferentiated; or grades I, II, and III, respectively) has proved to be the most reproducible and the best suited to predict survival.

The microscopic type or subtype of a tumor is related to its

grade; for instance, embryonal rhabdomyosarcoma is by definition a high-grade neoplasm. The significance of a parameter depends greatly on the type of lesion. Marked variations may exist between various areas of the same tumor, and accurate grading requires representative, well-fixed, and well-stained histologic material. Tumors for which microscopic grading correlates with prognosis include soft tissue sarcomas,<sup>7</sup> squamous cell carcinomas of various organs, breast carcinoma,<sup>8</sup> and prostatic adenocarcinoma.<sup>9</sup>

## PRECANCEROUS CONDITIONS AND CARCINOMA IN SITU

Probably no field in tumor pathology is more confusing and controversial yet as important clinically as that of the so-called *precancerous conditions*. The term embraces all the morphologically recognizable disorders thought to predispose a person to the development of malignancy. Because cancer is a multistep process in most instances, the concept can be expressed as the morphologic identification of the various steps that precede the development of a full-blown malignancy.<sup>10</sup> It is unfortunate that the terminology used for conceptually and often morphologically comparable processes differs so much depending on the organ systems in which they develop. It includes terms such as *dysplasia*, *atypical hyperplasia*, *atypical proliferation*, *intraepithelial neoplasia*, and *carcinoma in situ*. In some organs, such as the stomach, the terms *severe dysplasia* and *carcinoma in situ* are used synonymously; in others, such as the uterine cervix, a distinction has been made between them. Conceptually, hyperplasia and atypical hyperplasia are reversible processes, but carcinoma in situ is not.

Although the continuum from hyperplasia to neoplasia can be convincingly demonstrated, the precise boundaries between them cannot be determined with an acceptable degree of accuracy. Because of this difficulty, the alternative designation *intraepithelial neoplasia* has been advanced. The proponents of this terminology defend it on the grounds that the difference between the terms of *dysplasia* or *atypical hyperplasia* and *carcinoma in situ* (CIS) gives the morphologic spectrum a semantic dividing point that is far sharper in words than in the histologic images.<sup>11</sup> Instead, they maintain, a term such as *intraepithelial neoplasia* coupled with a grading system to indicate increasing degrees of severity can accomplish the following: emphasize the biologic and clinical unity of the two apparent conditions of dysplasia and CIS; remove from the pathologist the difficult and subjective task of distinguishing between severe dysplasia and CIS; indicate that although the grading of an intraepithelial lesion is of collective prognostic value, such grading offers no grounds for assuring a patient that this abnormality will or will not develop into an invasive carcinoma; allow for a unity of therapeutic approach and "prevent the state of affairs where a diagnosis of carcinoma in situ is regarded as a definite and often urgent indication for treatment whilst one of dysplasia, often differentiated from carcinoma in situ on relatively flimsy and uncertain pathologic grounds, is either not treated adequately or is ignored."<sup>11</sup>

The term *carcinoma in situ* (or the analogous term for melanocytic lesions, *melanoma in situ*) is being used with decreasing frequency in most sites. The statements that follow reflect in an eloquent fashion this increasingly popular point of view:

The best terminology for atypical intraepithelial lesions should accurately reflect the limitations of our knowledge about the process of carcinogenesis: 1) our inability to distinguish between atypical hyperplasia (reversible) from intraepithelial neoplasia (irreversible) and 2) our lack of criteria to recognize fully cancerized cells capable of invasion. The terminology should be simple and reproducible so that therapeutic protocols may be applied. It seems best to speak of atypical intraepithelial proliferation.<sup>12</sup>

The multi-step theory of neoplasia makes the definition of a malignancy in situ easy. Malignancy in situ is a state in which one or several cells and their progeny have acquired the potential to invade and to metastasize, but have not as yet exercised this option. The problem is that we cannot recognize these fully transformed cells until they actually invade the underlying tissue. In other words, a true malignancy in situ is not diagnosable at the present time by microscopy.<sup>13</sup>

The diagnosis of carcinoma in situ (melanoma in situ, malignancy in situ) is a contradiction in terms, the prototype of an oxymoron.<sup>14</sup>

## METHODS IN ONCOLOGIC PATHOLOGY

The standard procedure for the pathologic evaluation of tissue specimens for tumors is (and is likely to remain for a long time) their examination under a light microscope after formalin fixation, paraffin embedding, and staining of the sections with hematoxylin-eosin. This technique has proved one of the most durable in the medical laboratory and has remained essentially unchanged—except for the automation of some of the steps—for more than half a century. Although it has some drawbacks, it offers considerable advantages over the many alternatives that have been proposed over the years: it is quick, inexpensive, suitable for most situations, and comparatively easy to master. Most important, it allows an accurate microscopic diagnosis in most cases. However, it cannot answer all of the questions that a given case may pose at the plain diagnostic level. It often proves insufficient when dealing with etiologic, histogenetic, or pathogenetic questions, or when trying to predict the responsiveness of a given tumor to one type of therapy or another. As a consequence, an increasingly sophisticated array of "special" techniques has evolved in an attempt to deal with these matters in a more effective fashion.

### SPECIAL FIXATIVES

Buffered 10% formalin remains the standard fixative. A mercuric chloride-based fixative generally known by the abbreviation B5 currently is preferred over formalin for the study of hematolymphoid malignancies, mainly because of the superior cytologic details it provides. Zenker's solution is another mercuric chloride-based fixative and provides nearly identical results.

Several attempts have been made to introduce fixatives that are equally satisfactory for light microscopic and ultrastructural examination, but on the whole these have been unsuccessful.<sup>15</sup> Whether more recent attempts to devise fixatives compatible with routine use but providing better antigenic preservation than formalin (for the purposes of immunohistochemical evaluations) will be better accepted remains to be seen.

### SPECIAL STAINS

Of the extensive battery of special stains listed in the manuals on histologic techniques, the tumor pathologist will find that only a few are of real diagnostic utility. This is particularly the case since the advent of immunohistochemistry, with its superior sensitivity and specificity.

The special stains most commonly used for human tumors are the following:

1. Periodic acid-Schiff (PAS) stain. This stain—one of the few in which the precise chemical nature of the reaction is known—demonstrates glycogen (in a specific fashion when used with a diastase-digested control) and is therefore routinely used for the study of Ewing sarcoma, which contains this polymer in large amounts. It also demonstrates various types of mucosubstances. Furthermore, it is the stain of choice for the demonstration of the intracytoplasmic granules that characterize the rare soft tissue neoplasm known as alveolar soft part sarcoma.
2. Silver (argentaffin and argyrophil) stains. The argentaffin reaction depends on the presence in the tissue of a substance that reduces silver salts. This substance is often of the phenolic group, which includes catecholamines or indolamines. Tumors that are typically argentaffin include carcinoid tumors and paragangliomas. In the argyrophilic reaction, an extraneous reducing agent is added to precipitate the silver. For all its pitfalls, it remains the best nonimmune light microscopic technique for the detection of neuroendocrine differentiation in tumors. Numerous technical variations have been proposed, of which we prefer the unmodified Grimelius' technique.<sup>16</sup>
3. Trichrome stains. The principal value of this family of stains consists in the evaluation of type and amount of extracellular material, particularly collagen. The three tissue structures demonstrated by the various dyes comprising this stain are nuclei, cytoplasm, and collagen, the latter being the only one having some degree of specificity. Despite assertions to the contrary, it is not useful to distinguish fibroblastic from smooth muscle or peripheral nerve tumors.
4. Phosphotungstic acid-hematoxylin (PTAH) stain. This stain traditionally has been used for the demonstration of intracytoplasmic filaments, but has been largely superseded by immunohistochemical techniques.
5. Stains for neutral lipids. Of these stains, oil red O is most commonly employed. In tumor pathology, the utility of fat stains is minimal. These stains are of no use in the differential diagnosis of liposarcoma; many liposarcomas contain little or no fat, whereas many nonadipose tissue neoplasms exhibit considerable amounts of fat, probably as a result of degenerative changes.
6. Mucin stains. The combination of Alcian blue and PAS is probably the most inclusive mucin stain, because it demonstrates mucosubstances of neutral, slightly acidic, and highly acidic types. These stains are useful as a sign of glandular differentiation in epithelial tumors. When combined with enzymatic digestion with hyaluronidase, they are of some utility in the differential diagnosis of soft tissue tumors, because cartilaginous neoplasms usu-

ally contain sulfated (hyaluronidase-resistant) mucins, in contrast to most other myxoid neoplasms.

### ENZYME HISTOCHEMISTRY

Enzyme histochemistry is of little use in tumor diagnosis. The only techniques used with some frequency are those for chloroacetate esterase (Leder's stain, for cells of the myeloid series and mast cells),<sup>17</sup> acid phosphatase (for cells of the histiocytic series),<sup>18</sup> alkaline phosphatase (for endothelial cells and germ cells),<sup>19</sup> and the DOPA reaction (for melanocytes and related melanin-producing cells).

Because enzymes are proteins and therefore immunogenic, they can also be demonstrated with immunohistochemical techniques even when no longer active.<sup>20</sup>

### TISSUE CULTURE

Pioneer work carried out in the Department of Pathology at Columbia-Presbyterian Hospital in New York City showed that histogenetic clues can sometimes be obtained from primary cultures of human tumors, taking advantage of the fact that tumor cells can express features of differentiation *in vitro* that are not easily appreciable *in vivo*.<sup>21</sup> The best known example is neuroblastoma, which grows neurites within 24 hours of having been placed in culture medium. Another is melanoma, which can make abundant melanin *in vitro* while being totally amelanotic *in vivo*. In some instances, the tumor differentiation has been induced *in vitro* by the addition of an exogenous agent such as cyclic AMP.<sup>22</sup> The most interesting recent example of this phenomenon is the neural differentiation that has been obtained in Ewing sarcoma of bone by the addition of AMP or TPA to the medium.<sup>23</sup> The most useful application of short-time tissue culture techniques is in the differential diagnosis of small round cell tumors of infancy. However, at a practical and strictly diagnostic level, the utility of tissue culture techniques remains limited.<sup>24</sup>

### ELECTRON MICROSCOPY

The use of electron microscopy in tumor diagnosis has diminished considerably since the advent of immunohistochemistry. However, it remains a powerful tool when used selectively and intelligently after a differential diagnosis has been formulated at the light microscopic level.<sup>25-30</sup>

Electron microscopy can contribute to the differential diagnosis between carcinoma, melanoma, and sarcoma; adenocarcinoma and mesothelioma; anterior mediastinal tumors (i.e., thymoma, thymic carcinoid, malignant lymphoma, seminoma); small round cell tumors (i.e., Ewing's sarcoma, embryonal rhabdomyosarcoma, malignant lymphoma, neuroectodermal tumors); and spindle cell tumors of soft tissues (i.e., muscular, pericytic, schwannian). It is also useful in detecting neuroendocrine differentiation in tumors (through the demonstration of dense core secretory granules), in identifying the cells of histiocytosis X (through the finding of Birbeck granules), and in confirming a diagnosis of alveolar soft part sarcoma (through the demonstration of membrane-bound intracytoplasmic crystals).

The main limitations of electron microscopy as a diagnostic tool relate to sampling (only a small portion of the tumor can

be studied), paucity of truly specific ultrastructural features, and the ever-present danger of misinterpreting entrapped nonneoplastic elements as belonging to the tumor.

### IMMUNOHISTOCHEMISTRY

The immunohistochemical method has contributed more than any other special technique to the histopathologic diagnosis of tumors. Basically, it consists of the application of immunologic principles and techniques—with their remarkable degree of specificity and sensitivity—to the study of cells and tissues.<sup>31-33</sup> It can be applied to routinely processed material (even if stored for long periods) and allows accurate correlations with the traditional morphologic parameters. It is compatible with most fixatives and is feasible even in material that has been decalcified.<sup>34</sup> It can also be applied to cytologic preparations and to electron microscopy, and can be used in conjunction with more conventional techniques (such as silver stains) in the same section.

For all its attributes, immunohistochemistry is fraught with numerous potential pitfalls. False-negative results can occur because of inappropriateness, denaturation, or wrong concentration of the antibody; loss of antigen through autolysis or diffusion; or presence of this antigen at a density below the level of detection. False-positive results can result from cross-reactivity with other antigens; nonspecific binding of the antibody to the tissue; entrapment of normal tissues by the tumor cells; and release of soluble proteins from the cytoplasm of normal cells invaded by the tumor, with permeation of the interstitium followed by nonspecific absorption or possibly phagocytosis by the tumor cells.<sup>35,36</sup> Other factors that have resulted in misinterpretations include the anomalous positive stains caused by ectopic antigen expression and hitherto unrecognized cross-reactions. Many markers thought to be specific for a certain cell, tissue, or tumor have proved to be shared by other tissues or tumors, and proper controls must therefore be used. An ingenious multitumor tissue block has been devised to test several tumor types in a quick and inexpensive fashion.<sup>37</sup>

The list of antigens that has been detected with immunohistochemistry is very large. Theoretically, any substance that is antigenic and whose antigenicity is at least partially retained in tissue sections can be demonstrated with this technique. The most important immunohistochemical markers in diagnostic tumor pathology are the following:

1. *Intermediate filaments.* Keratin serves as a marker of epithelial differentiation, vimentin as a generic marker of mesenchymal cells, desmin as a marker of muscle differentiation, neurofilaments as a marker of neural differentiation, and glial fibrillary acidic protein as a marker of glial differentiation.<sup>38-43</sup> Twenty or more antigenically distinct subclasses of keratin have been described having a tissue-related distribution in the various epithelia that is often recapitulated by the respective neoplasms.<sup>44</sup>
2. *Markers of muscle differentiation.* In addition to desmin, these markers include smooth muscle actin, skeletal muscle actin, myoglobin, and the product of *MYOD1* gene. All but the first are specific for striated muscle.<sup>45-48</sup>

3. **Lymphoid markers.** A huge number of monoclonal antibodies are available against the multitude of cell surface markers present in lymphocytes and related hematopoietic cells, sometimes as an expression of cell type or subtype and sometimes as a sign of a functional status, such as activation. Of those detectable in formalin-fixed, paraffin-embedded material, the most useful are leukocyte common antigen (LCA, a pan-lymphoid marker), L-26 (a pan-B-cell marker), UCHL1 (a pan-T-cell marker), Ber-H2 (analogous to Ki-1 and expressed by the Reed-Sternberg cells of Hodgkin's disease and the cells of so-called anaplastic large-cell lymphoma), and LeuM1 (expressed in Reed-Sternberg cells and in some T-cell lymphomas).<sup>48</sup>
4. **S-100 protein.** Although more ubiquitous than originally believed, the demonstration of this marker is of use in the diagnosis of melanocytic, schwannian, and cartilaginous neoplasms.<sup>50,51</sup>
5. **HMB-45.** This is a helpful although not entirely specific marker of activated melanocytes, particularly those of malignant melanoma.<sup>52</sup>
6. **Carcinoembryonic antigen (CEA).** This marker is mainly expressed by epithelial neoplasms, particularly those of glandular nature. Its main use is in the differential diagnosis between adenocarcinoma (usually positive) and malignant mesothelioma (nearly always negative).<sup>53</sup>
7. **Epithelial membrane antigen (EMA).** This is a general marker for epithelial differentiation.<sup>54</sup>
8. **Chromogranin, neuron-specific enolase, and synaptophysin.** These are markers of neural or neuroendocrine differentiation.<sup>55-57</sup>
9. **Various peptide hormones (e.g., gastrin, insulin, calcitonin, ACTH).** These are markers of specific endocrine cell types.
10. **Organ-related markers,** such as thyroglobulin (for thyroid follicular cells),<sup>58</sup> prostatic specific antigen (for prostatic epithelium),<sup>59</sup> and GCDFP-15 (for apocrine epithelium and breast carcinoma).<sup>60</sup>
11. **Human chorionic gonadotropin (HCG),  $\alpha$ -fetoprotein, and human placental lactogen.** These are markers for various germ cell malignancies.
12. **Factor VIII-related antigen and Ulex europaeus I lectin.** These are markers of endothelial cells.<sup>61,62</sup>
13. **Type-IV collagen and laminin.** These are markers for basement membrane deposition.<sup>63</sup>
14. **Markers for microorganisms,** including viruses (e.g., cytomegalovirus, human papillomavirus), bacteria, fungi, and parasites.
15. **Hormone receptors.** Reliable monoclonal antibodies are available for the detection of estrogen, progesterone, and androgen receptors in tissues (see Chap. 40).<sup>64-67</sup>

### FLOW CYTOMETRY

Flow cytometry consists of the simultaneous measurement of several parameters while a suspension of cells flows through a beam of light past stationary detectors. It allows the analysis of 5000 to 10,000 cells per second for features such as cell size, cytoplasmic granularity, cell viability, cell cycle tissue, DNA content, surface marker phenotype, and enzyme content.

The main limitation of the technique is that cells must be in a single-cell suspension to be analyzed. This requirement is easily achieved in blood and other fluids, but obtaining satisfactory samples from nonhematopoietic solid tumors is more difficult. However, suitable techniques have been developed for most tumors, including a preparation of nuclear suspensions recovered from thick sections of routine formalin-fixed paraffin-embedded tissue blocks.<sup>68,69</sup> The main clinical uses of flow cytometry in solid tumors are as follows:<sup>70-73</sup>

- To support a diagnosis of malignancy when the morphologic changes are equivocal, through the demonstration of an aneuploid cell population
- To subclassify lesions of borderline malignancy
- To provide prognostic information independent of stage and grade
- To monitor response to therapy
- To document the appearance of tumor relapse
- To help determine whether two anatomically separate tumors of similar histology (whether synchronous or metachronous) are independent.

### NUCLEIC ACID HYBRIDIZATION

Hybridization techniques are based on the application of recombinant DNA technology. The labeling of the probes can be done with radioactive or nonradioactive compounds (e.g., bromodeoxyuridine).<sup>74-79</sup> Visualization of mRNA in tumor cells is being used increasingly to detect specific gene expressions, particularly those encoded by oncogenes and neuroendocrine-related genes.<sup>80</sup>

The two major techniques for hybridization of nucleic acids are the transfer or blotting methods (i.e., Southern blotting for DNA, Northern blotting for RNA, and Western blotting for proteins) and in situ hybridization. The latter technique allows the visualization of cellular DNA or RNA in tissue sections, single cells, or chromosome preparations. The advantages of in situ hybridization over the transfer method are that it can be applied to small samples (in some instances even to paraffin-embedded material) and that it allows detection of the reaction product in specific cell types and subtypes or even subcellular sites. It allows a close correlation between the reaction and the morphologic appearance of the cells in which the reaction has occurred, a feature that represents the *desideratum* of any special technique in pathology.

Two important applications of nucleic acids hybridization in tumor pathology are in the evaluation of oncogenes and in the study of gene rearrangements in lymphoid diseases.

### OTHER METHODS FOR ANALYSIS OF CELL PROLIFERATION

In addition to flow cytometry, several other methods exist for the evaluation of the degree of cellular proliferation.<sup>81</sup> These include mitotic count, thymidine labeling, microspectrophotometric analysis, and histochemical or immunohistochemical detection.

#### Mitotic Count

The older and most widely used method for evaluating cell proliferation is mitotic count. It is usually applied to routinely

processed sections, the standard figure quoted being the number of mitoses in ten consecutive high-power fields (usually the combination of a 10X eyepiece and a 40X objective). Its most useful application is in the evaluation of mesenchymal neoplasms, particularly uterine smooth muscle tumors. Despite its apparent objectivity, results vary considerably depending on the thickness of the section, fields chosen, density of tumor cells (in absolute terms and in relation to nonneoplastic elements), type of microscope used, delay in fixation, and observer's variability in the identification of mitotic figures. Some of these handicaps can be eliminated by expressing the number of mitotic figures as percentages of tumor cells present.<sup>82</sup>

### Thymidine Labeling

Thymidine labeling of fresh tumor tissue is followed by fixation, paraffin-embedding, and radioautography. The labeled nuclei are those that have incorporated the tritiated thymidine and are therefore in the S phase (DNA synthesis). An alternative (nonradioactive) marker is bromodeoxyuridine, a thymidine analog that is incorporated into nuclear DNA during the S phase and is detectable immunohistochemically.<sup>83,84</sup>

### Microspectrophotometric Analysis

This analysis is performed by staining tissue sections with the Feulgen reaction (which is specific for DNA) and determining the DNA content (expressed in arbitrary units) in a microspectrophotometer.

### Histochemical or Immunohistochemical Detection

Histochemical or immunohistochemical analysis can detect substances related to cell growth and division. These include the following: Ki-67, a monoclonal antibody that binds to nuclear antigens expressed by cells in the proliferative phases G<sub>1</sub>, G<sub>2</sub>, M, and S;<sup>85-87</sup> proliferating cell nuclear antigen (PCNA, cyclin), one of several cycle-related nuclear proteins that is maximally elevated in late G<sub>1</sub> and S phases of proliferating cells;<sup>88,89</sup> and nucleolar organizing region-associated proteins (AgNor), a marker of cell activation demonstrable with a simple silver technique.<sup>90-92</sup>

## IMAGE ANALYSIS

The quantitative analysis of images has been explored as a method to generate prognostic and perhaps diagnostic information on tumors and other lesions. It can objectively quantify individual cytologic criteria, such as nuclear size, variation in size and shape, and degree of roundness.<sup>93-95</sup> It can also be applied at the architectural level to measure the total surface (and, by inference, the volume) of tumor involving a given site. For instance, in a study of prostatic carcinoma diagnosed on transurethral resection, the area of tumor as measured by image analysis was a better predictive factor for survival than the conventional method of calculating the percentage of involved chips.<sup>96</sup> Image analysis can also be used to measure immunohistochemical stainings for hormone receptors or any other markers.<sup>97</sup>

## DIAGNOSTIC CYTOLOGY

Diagnostic cytology (cytopathology) deals with the morphologic examination of individual cells (as opposed to tissues in histopathology). Specimens used for this purpose are obtained in one of three ways: by exfoliation or desquamation from an epithelial surface (such as cervical smears obtained with a spatula or brush); by fluid from a body cavity, whether obtained spontaneously (i.e., urine) or by aspiration (i.e., pleural or ascitic fluid); and by fine-needle aspiration of solid lesions. Whatever the source of the material, the cytologic preparation is then spread on a glass slide, fixed, and stained. The Papanicolaou's stain is the one most commonly used, because of the greater nuclear details it is supposed to provide.

The criteria for the evaluation of cytologic specimens vary from organ to organ, but the basic criteria are common and relate to specific features of the nucleus and cytoplasm. Abnormalities in nuclear structure are grouped under the term *dyskaryosis* and include coarse or dense-granularity, hyperchromasia, abnormal (large, multiple, or irregularly shaped) nucleoli, and variations in the shape and size of the nuclei. Abnormal mitoses may also be present. Two other common features of malignant tumor are *pleomorphism* (a pronounced variation in shape) and *anisocytosis* (a pronounced variation in size). The nuclear-cytoplasmic ratio generally increases, largely because of the increased nuclear size but also (in many cases) because of the smaller amount of cytoplasm compared with the corresponding normal cells. The cell-to-cell relations are likely to be altered.

### FINE-NEEDLE ASPIRATION BIOPSY

The cytologic procedure generally known as *fine-needle aspiration biopsy* had its inception more than 50 years ago at Memorial Hospital in New York City.<sup>98,99</sup> All the basic rules that still govern the performance of this method were carefully set down in articles published by Dr. Fred Stewart. For some reason, the popularity of the procedure declined at that institution, to be revived decades later in Europe (particularly in Scandinavian countries) by clinicians, until achieving popularity on a worldwide basis.<sup>100-103</sup>

Aspiration biopsy is most useful in tumor diagnosis. Experience is needed to obtain optimal results, which vary markedly depending on seemingly trivial items such as the needle size and design, the way the needle is inserted and withdrawn from the needle track, the direction of the smear preparation, the type of slide (plain or frosted), and the staining method. It has been stated that mastery of the interpretation of aspiration biopsy cytology lies in the ability to translate cytologic appearances into tissue patterns with diagnostic significance. Reliance on individual cell features to the exclusion of pattern, as practiced by cytopathologists having little background knowledge of tissue pathology, decreases diagnostic accuracy and reflects a lack of understanding of the method.

An important and much discussed issue is the reliability of the technique, usually measured in terms of false-negative and false-positive results. These are difficult figures to obtain and to evaluate for several reasons, including the standards against which they are compared (i.e., histopathologic diagnosis versus clinical outcome), the impact of actually performing and interpreting the aspirate as opposed to receiving



a specimen taken by others for processing and review, and the statistical methods employed.

The main sites in which fine-needle aspiration is used are the breast, lymph nodes, salivary glands, thyroid, lung, intraabdominal organs, prostate, bone, and soft tissue.

### **Breast**

The breast has been one of the preferred sites for the use of the fine-needle aspiration technique from its inception. The reported series list 10% or fewer false-negative figures for breast carcinoma and a negligible number of false-positive diagnoses. In the past, most studies in this field were directed at clinically apparent breast masses. Recently, Swedish investigators have developed a stereotactic device for the sampling of subclinical, mammographically detected breast lesions. The technique has been adopted on a trial basis by several groups in the United States, but it is too early to make a statement about its effectiveness.

### **Lymph Nodes**

The original use of the technique in lymph nodes was in the diagnosis of leukemia, malignant lymphoma, and infectious diseases. Its main use now is to confirm a clinical diagnosis of metastatic carcinoma and to determine the type and site of the primary tumor whenever indicated. The sensitivity for tumor diagnosis is more than 95%, and the specificity for the absence of malignant tumor is more than 97%.

### **Salivary Glands**

Benign mixed tumor is the most common neoplasm of the major salivary glands and is also the one most easily recognized in specimens from fine-needle aspiration. Warthin's tumor is also readily identifiable. Adenoid cystic carcinoma gave rise to serious interpretive problems in the past, but with the establishment of better cytologic patterns, it is recognized with ease in most instances. Problems still arise in recognizing acinic cell carcinomas and mucoepidermoid carcinoma, because of their well-differentiated nature.

Most salivary gland swellings are inflammatory in nature. Cytologically, most can be recognized easily because of the admixture of normal-appearing ductal and acinar cells and inflammatory cells.

### **Thyroid**

The most common application of the fine-needle aspiration technique is in the evaluation of the single cold nodule. In deciding whether the nodule represents a true neoplasm or part of a nodular hyperplasia (adenomatoid goiter), the sensitivity and specificity of the technique are more than 90% accurate. In determining the tumor type, cytology is accurate in the identification of papillary carcinoma (including most of its variants), poorly differentiated and undifferentiated carcinoma, and medullary carcinoma. The main difficulty rests in distinguishing between follicular adenoma and low-grade (i.e., well-differentiated, minimally invasive) follicular carcinoma. This is because the distinction is largely based on capsular and vascular invasion, two criteria that cannot be

identified cytologically. However, because statistically follicular carcinomas are more likely to be more cellular and their nuclei more hyperchromatic than those of adenoma, a reasonably accurate "index of suspicion" for malignancy has been devised.

In practice, the three most common diagnoses made in thyroid fine-needle aspiration are nonneoplastic disorder (nodular goiter or thyroiditis), papillary carcinoma, and follicular neoplasm (not otherwise specified). The latter two diagnoses generally indicate the need for an operation.

### **Lung**

Improvements in fluoroscopy have made the fine-needle aspiration procedure in the lung accurate and cost-effective, allowing the diagnosis of small lesions. In most series, false-positive results are fewer than 1%, but false-negative results occur in fewer than 10% of cases. For lung carcinoma, cytology allows typing just as accurately as histopathology, particularly in regard to the tumor for which typing carries the greatest practical importance (i.e., small cell carcinoma).

Transthoracic fine-needle aspiration is perhaps the only aspiration procedure associated with occasional but significant complications. These include pneumothorax (about 10%), air embolism, and hemorrhage.

### **Intraabdominal Organs**

More fine-needle aspirations are being performed in tumors in the abdominal cavity and retroperitoneum, including such sites as the liver, biliary tree, pancreas, adrenal, kidney, and retroperitoneal soft tissues. This has been related to the better localization of the lesion as a result of computed tomography, magnetic resonance imaging, and ultrasonography. For carcinomas of the ovary and uterus (usually recurrent in the pelvis), aspiration procedures can be carried out transvaginally or transrectally. The diagnosis of recurrent carcinoma involving the pelvis is easily made in most cases.

### **Prostate**

Use of fine-needle aspiration in the prostate is controversial. Although the method is performed easily in an office setting, is relatively free of complications, is repeatable during the same visit or subsequent visits, and is cost-effective and reliable, urologists in the United States are reluctant to use the technique on a large scale. In the reported series, accuracy has ranged from 63% to 91%.

### **Bone and Soft Tissue**

For bone lesions, the main use of aspiration cytology has been to identify metastatic tumors, with excellent results in terms of sensitivity and specificity. High-grade soft tissue sarcomas are identified with ease on cytologic preparations, because they usually lack the cell cohesiveness of metastatic carcinomas. The cell pattern may suggest a specific diagnosis, such as liposarcoma or leiomyosarcoma.

Cytologic material can be subjected to most of the special techniques that have been introduced over the years in histopathology. These include conventional special stains (e.g.,

PAS or mucicarmine), immunohistochemistry, electron microscopy, cytogenetics, molecular diagnostics, ploidy determination, and image analysis.<sup>104-106</sup> In image analysis, several efforts are being made to develop automated screening devices for the detection of abnormal cells in cytologic specimens from the female genital tract.<sup>107</sup> These devices use quantitative parameters to define limits of normality for the digital analysis of smears or monolayers. These instruments have the potential to serve as prescreening or rescreening devices for quality assurance, but large-scale prospective testing is required to decide how much of this potential can be realized.

## INTRAOPERATIVE CONSULTATION

Intraoperative consultation (often referred to as *frozen section*) is one of the most important activities that the tumor pathologist performs. Introduced in this country by Welch in 1891 and developed for intraoperative pathologic diagnosis at the Mayo Clinic by Wilson and MacCarthy in 1905,<sup>108</sup> it is now used routinely in all pathology laboratories. It requires extensive experience, a good background in clinical medicine and anatomic pathology, sound judgment, and a keen awareness of the limitations of the method.

The reasons for doing a frozen section vary from organ to organ, but the basic indications remain the same. The three legitimate purposes of a frozen section are to establish the presence and nature of a lesion, to determine the adequacy of surgical margins, and to establish whether the sample obtained is adequate for the purpose for which it was taken (i.e., for diagnosis or for special procedures such as hormone receptors or cell markers). If the result of the procedure will not influence in any way the subsequent course of the operation, the procedure is not indicated.

The overall accuracy of the procedure has been tested on numerous occasions and is consistently very high.<sup>108-112</sup> The Association of Directors of Anatomic and Surgical Pathology has estimated that an acceptable accuracy threshold is 3% (as measured by the number of cases in which a major disagreement existed between the frozen section and the permanent diagnosis).<sup>113</sup> Reasons for these discrepancies include mistaken interpretation of the slide, inadequate sampling of the gross specimen received, inadequate technical quality of the material, and lack of crucial clinical information. The first two together account for about 90% of the mistakes.

To carry out the procedure effectively, the pathologist should be thoroughly briefed on the patient's clinical history. Ideally, the surgeon and the pathologist should discuss the case beforehand, and the pathologist should review any previous slides on the case. The standard technique involves sampling of the tissue received, freezing, cutting of sections in a cryostat, staining of those sections with hematoxylin-eosin or an equivalent stain, and examination under the microscope. Ideally, this procedure takes about 5 minutes per specimen, although extra time must be allowed if multiple sections or specimens need to be examined on one case or if several cases are submitted at the same time.

Some groups have resurrected cytology to supplement and sometimes replace the frozen-section procedure as a way to provide an intraoperative diagnosis, a technique described as early as 1927. The proposal is attractive on several grounds,

one of them being the considerable amount of time saved (it takes 1 minute or less per slide and 5 to 10 minutes for a frozen section).<sup>114,115</sup> Although there are some specimens for which this technique is not feasible (e.g., surgical margins or assessment of depth of invasion), there are many others in which it can provide as much information as the frozen section (or sometimes even more) and in a more expeditious fashion. Additional advantages are that the tissue is better conserved for subsequent permanent sections (a feature particularly important with small samples) and that it provides more thorough sampling of large, multiple, or highly necrotic specimens.

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New Antibody

## Diagnostic Utility of Microphthalmia Transcription Factor in Malignant Melanoma and Other Tumors

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**Summary:** The diagnosis of malignant melanoma is sometimes challenging. Immunohistochemistry for specific markers of melanocytic differentiation such as HMB-45 and Melan-A can be very valuable in proving melanocytic differentiation in poorly differentiated or spindled forms of melanoma. Microphthalmia-associated transcription factor (Mitf) is the most recently described and the only nuclear melanocytic marker. This article reviews the biology of Mitf and those published studies that have addressed its diagnostic sensitivity and specificity. Mitf may be very valuable for the diagnosis of melanoma, including desmoplastic variants; melanocytic soft tissue tumors, such as clear cell sarcoma; and the unusual group of tumors that show combined melanocytic and myoid differentiation, the perivascular epithelioid cell family of tumors (PEComas). **Key Words:** Microphthalmia transcription factor—Malignant melanoma—Perivascular epithelioid cell—Clear cell sarcoma—Diagnosis—Monoclonal antibodies—Immunohistochemistry

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Each year, approximately one million Americans are diagnosed with skin cancer, including approximately 35,000 cases and 7200 deaths due to malignant melanoma (1). Despite the presence of numerous international and national health care programs promoting behavioral changes and incorporating skin cancer control, the practicing surgical pathologist has seen an increase in the number of melanoma specimens in the past decade. The diagnosis and staging of typical cutaneous melanoma are generally accomplished easily, using only histologic techniques. However, atypical presentations of cutaneous melanomas and melanomas presenting in noncutaneous sites present diagnostic challenges and require the use of adjunct diagnostic techniques, such as paraffin immunohistochemistry. There are two situations

in which immunohistochemistry may be particularly valuable in the diagnosis of melanoma: poorly differentiated pleomorphic or epithelioid melanomas, which may be mistaken for a carcinoma or anaplastic lymphoma, and desmoplastic malignant melanomas, which may be confused with dermal scars, tumors of neuroectodermal or nerve sheath derivation, atypical fibroxanthoma/superficial malignant fibrous histiocytomas, and sarcomatoid squamous cell carcinomas. Immunohistochemistry for specific melanocytic markers, such as HMB-45, Melan-A, and tyrosinase, may also play a valuable role in the diagnosis of unusual sarcomas with melanocytic differentiation (e.g., clear cell sarcoma, melanotic schwannoma) and in the rare group of tumors that display combined melanocytic and muscle differentiation, the perivascular epithelioid cell family of tumors (PEComas; e.g., angiomyolipoma and related tumors). Although a number of specific markers of melanocytic differentiation are available, including mAb HMB-45

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and antibodies to Melan-A and tyrosinase, none of these markers is entirely sensitive for the diagnosis of even typical melanomas. The situation is even worse with regards to desmoplastic malignant melanomas, which are typically positive only for vimentin and S100 and negative with specific melanoma markers (2). The search for new melanoma markers therefore continues.

There now exist commercially available antibodies to microphthalmia-associated transcription factor (MiTF), which is a DNA-binding protein that plays a critical (but poorly understood) role in melanocyte development. In humans, MiTF consists of at least four isoforms, which differ at their amino-termini and expression patterns (3). Two isoforms (A and B) are present in retinal pigment epithelium (RPE), cervical carcinoma cells, and melanoma cells. Isoform H is present in RPE and cervical cancer cells but not melanoma cells. Finally, isoform M is present only in melanoma cells (4). In humans, mutations of MiTF are responsible for Waardenburg syndrome type 2a, which is characterized by pigmentation abnormalities and sensorineural deafness due to the absence of melanocytes from the stria vascularis of the inner ear (5). Knockout of MiTF in mice also disrupts osteoclastogenesis and results in osteopetrosis (6). The commercially available clone C5 detects both mouse and human MiTF, whereas the commercially available clone D5 detects only human MiTF (7). A "cocktail" of C5 and D5 is also commercially available; this recognizes both melanocytes and osteoclasts.

Microphthalmia-associated transcription factor has been shown to be a highly sensitive and specific marker of benign cutaneous nevi and epithelioid malignant melanoma, being expressed in all nevi studied and between 88% and 100% of primary and metastatic epithelioid melanoma (7-11). These results compare favorably with HMB-45, Melan-A, and tyrosinase (12-14). The situation is somewhat less clear, however, with regards to the utility of MiTF in the diagnosis of desmoplastic malignant melanoma. Four studies have looked at MiTF expression desmoplastic melanoma, with the findings of MiTF expression in 35% (10), 55% (8), 3% (11), and 7% (9) of cases, respectively. As discussed below, these differences in rates of positive desmoplastic melanoma are most likely related to case selection criteria. The 35% and 55% positivity rates cited in the first two studies are quite a bit higher than what is generally seen with HMB-45 or Melan-A and suggest that MiTF is at least equal to, and quite possibly a good deal better than, these two older markers in this difficult diagnostic situation.

Microphthalmia-associated transcription factor expression has with one exception been found to be highly, but not absolutely, specific for melanocytic differentia-

tion. Two of five previous studies have found no MiTF expression in any nonmelanocytic tumor (7,9). Koch et al. (8) found focal MiTF expression in two neurofibromas and one purported atypical fibroxanthoma. Miettinen and coworkers (11) documented MiTF expression in normal histiocytes but not in any of over 200 non-melanomas studied. However, Busam et al. (10) reported MiTF expression in a significant percentage of dermal spindle cell tumors, including fibrous histiocytomas, fibrosarcomas, and leiomyosarcomas, as well as in various adenocarcinomas, germ cell tumors, lymphomas, and soft tissue neoplasms. MiTF expression has also been documented in a case of renal cell carcinoma and in one well-differentiated liposarcoma (15).

How can these differences be explained? All of these studies were performed using either the D5 clone or the C5+D5 cocktail and all used heat-induced epitope retrieval. None of the studies appears to have used the antibodies at an inappropriately high or low dilution, although the dilutions do vary slightly from study to study. The only methodological difference that stands out is the use of overnight primary antibody incubation in the study with the highest rate of nonmelanoma staining (10). It is conceivable that this additional incubation period either allowed for the detection of minimal amounts of MiTF present in perhaps a wide range of cell types or may have in fact been responsible for a cross-reactivity of the antibody with such epitopically related molecules as E(TFE)3, TFEB, and TFEC (9). The difference in the percentages of positively staining desmoplastic melanomas is probably best explained by case selection bias, in that Koch et al. grouped together typical desmoplastic and more cellular spindle cell melanomas, both of which had been submitted as possible sarcomas to a large sarcoma consultation service. The other studies used primarily typical, paucicellular dermal tumors. It has been the authors' experience that MiTF expression is far less common in small dermal desmoplastic melanomas than in those that form a distinct mass.

Microphthalmia-associated transcription factor expression is also commonly seen in two other groups of tumor, PEComas and soft tissue neoplasms with melanocytic differentiation. The PEComas are an unusual group of neoplasms that show combined features of melanocytic and myoid differentiation. Its members include relatively common entities such as angiomyolipoma and lymphangiomyomatosis, as well as far less common tumors, including clear cell "sugar" tumors of the lung, clear cell myomelanocytic tumors of the falciiform ligament, and occasional tumors of the pancreas and uterus (16-21). MiTF expression has been documented in between 75% and 100% of angiomyolipomas examined

(15,22) and in all clear cell myomelanocytic tumors (16). MiTF appears to be approximately equal to HMB-45 and Melan-A in the diagnosis of PEComas. However, one potential advantage of MiTF in small biopsies is that it appears to be more often positive in a large number (>50%) of cells in a given tumor. Additionally, as a nuclear marker, MiTF is largely free of the cytoplasmic artifacts that plague the immunostaining of biotin-rich and/or peroxidase-rich tissues such as kidney and liver (15). With regard to soft tissue neoplasms showing melanocytic differentiation, MiTF expression has been documented in the great majority of melanotic schwannomas, cellular blue nevi, and clear cell sarcomas (8,23).

In conclusion, MiTF appears to be the most sensitive currently available marker of benign melanocytic nevi and typical epithelioid melanoma. The sensitivity of MiTF for desmoplastic melanoma is significantly less than for epithelioid melanoma, but still better than HMB-45, Melan-A, or tyrosinase. Unlike HMB-45 and tyrosinase, however, MiTF expression does not appear to be absolutely specific for tumors with melanocytic differentiation, as noted above. MiTF, therefore, appears to be a highly promising new marker. The reader is cautioned, however, as with any new marker, to always interpret MiTF results within the context of an appropriate battery of positive and negative immunostains.

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# MLANA/MART1 and SILV/PMEL17/GP100 Are Transcriptionally Regulated by MITF in Melanocytes and Melanoma

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The clinically important melanoma diagnostic antibodies HMB-45, melan-A, and MITF (D5) recognize gene products of the melanocyte-lineage genes SILV/PMEL17/GP100, MLANA/MART1, and MITF, respectively. MITF encodes a transcription factor that is essential for normal melanocyte development and appears to regulate expression of several pigmentation genes. In this report, the possibility was examined that MITF might additionally regulate expression of the SILV and MLANA genes. Both genes contain conserved MITF consensus DNA sequences that were bound by MITF *in vitro* and *in vivo*, based on electrophoretic mobility shift assay and chromatin-immunoprecipitation. In addition, MITF regulated their promoter/enhancer regions in reporter assays, and up- or down-regulation of MITF produced corresponding modulation of endogenous SILV and MLANA in melanoma cells. Expression patterns were compared with these factors in a series of melanoma cell lines whose mutational status of the proto-oncogene BRAF was also known. SILV and MLANA expression correlated with MITF, while no clear correlation was seen relative to BRAF mutation. Finally, mRNA expression array analysis of primary human melanomas demonstrated a tight correlation in their expression levels in clinical tumor specimens. Collectively, this study links three important melanoma antigens into a common transcriptional pathway regulated by MITF. (*Am J Pathol* 2003; 163:333-343)

MITF, a bHLHZip (basic/helix-loop-helix/leucine-zipper) transcription factor, is essential for the development and maintenance of the melanocyte lineage.<sup>1</sup> MITF binds the canonical E-box promoter sequence CACGTG as well as the non-palindromic sequence CACATG.<sup>2,3</sup> The three major pigmentation enzymes tyrosinase, TYRP1, and

DCT all contain the MITF consensus binding site in their promoters and are thought to be transcriptional targets of MITF.<sup>2,4,5</sup> In humans, germline heterozygous MITF mutation produces, with variable penetrance, Waardenburg Syndrome IIA,<sup>6</sup> and Tietz syndrome,<sup>7,8</sup> manifesting pigmentation disturbances and deafness, the latter due to inner ear melanocyte deficiency.<sup>9</sup> In mice, the allele *Mitf<sup>ml</sup>* leads to complete absence of melanocytes in the homozygous state,<sup>10</sup> while mice homozygous for *Mitf<sup>ml</sup>* (*vittigo*) are born with a nearly normal coat color, but turn white over the first few months of life as melanocytes are lost, suggesting a role for MITF in both melanocyte development and in postnatal survival.<sup>11,12</sup> In contrast to various melanocytic markers such as melanin or c-kit whose expression may be lost or difficult to detect in malignant melanocytic lesions,<sup>13-15</sup> MITF expression is usually maintained in human melanoma specimens,<sup>16-19</sup> and MITF is increasingly used as a histopathological marker for melanoma diagnosis.<sup>20</sup>

PMEL17 is the human homologue of murine *silver*, a locus whose disruption produces a significant pigmentation phenotype which resembles a silver color.<sup>21</sup> Although the precise function of this gene (also known as GP100/SILV, hereafter referred to as SILV) remains to be ascertained, SILV localizes to an early melanosome trafficking compartment<sup>22</sup> and may function in melanosome structure,<sup>23</sup> biosynthesis of the melanin intermediate 5,6-dihydroxyindole-2-carboxylic acid (DHICA),<sup>24,25</sup> or morphogenesis of premelanosomes.<sup>26</sup>

MLANA/MART1 (hereafter referred to as MLANA) was cloned by two separate groups using melanoma reactive cytotoxic T lymphocytes to screen a cDNA library derived from melanoma cells.<sup>27,28</sup> MLANA expression is restricted to melanocytes, melanomas, and retinal pigment epithelium.<sup>27</sup> Its function remains to be fully elucidated, but it has recently been found to localize to vesicles, including melanosomes, suggesting a role in melano-

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some biogenesis.<sup>29</sup> Although near the loci for two murine coat color mutants, *MLANA* has been excluded as a candidate for either,<sup>30</sup> and its loss of function phenotype is currently unknown.

Aside from its role in pigmentation, *SILV* encodes antigenic epitopes which are recognized by multiple melanoma diagnostic antibodies including HMB-45, currently one of the most commonly used melanocytic markers for clinical melanoma diagnosis in humans.<sup>31,32</sup> The Melan-A and MART1 antibodies both recognize the *MLANA* gene product and are increasingly being used as markers for the diagnosis<sup>33-36</sup> and prognosis of melanoma.<sup>37</sup> Due to its early vertical (deep) growth phase, cutaneous melanomas exhibit a high propensity for local invasion and metastasis. Furthermore, melanoma may present as "metastasis of unknown primary" or relapse after considerable time intervals, often lacking differentiated features (such as pigmentation). The diagnosis of melanoma may therefore rely on combinations of immunohistochemical markers for diagnosis. Despite the common loss of many melanocyte-specific markers in melanoma, HMB-45 positivity has been seen in a high fraction of human melanoma specimens<sup>38,39</sup> and antibody staining for the melanocyte-melanoma lineage-specific antigen *MLANA* was shown to be positive in all primary malignant melanomas and nevi tested.<sup>40,41</sup> In subsequent studies, *MLANA* reactivity has been shown to largely overlap with tyrosinase<sup>42</sup> and HMB-45.<sup>33,43</sup> MITF (antibody D5) was also found to stain a high fraction of melanomas, with a pattern that included all HMB45-positive specimens.<sup>16</sup> A recent side-by-side examination of MITF, HMB-45, and Melan-A revealed high sensitivity and specificity for all three in melanoma diagnosis of cytologic specimens.<sup>44</sup>

Both *SILV* and *MLANA* have been identified as antigens recognized by tumor infiltrating lymphocytes,<sup>28,45-47</sup> leading to their use in vaccine protocols aimed at immunotherapy for melanoma.<sup>48-51</sup> Dramatic melanoma regressions were recently reported for patients undergoing intensive immunotherapy directed against *SILV* and *MLANA*.<sup>52</sup> In addition, it has been suggested that expression patterns of MITF and *MLANA* may correlate with specific clinical behaviors of melanoma, with higher expression of these markers associated with better prognosis.<sup>17,53-55</sup>

In this study, we demonstrate evidence of linkage between the expression patterns of the three melanoma diagnostic markers, MITF, *SILV* and *MLANA*. MITF consensus recognition sites were identified in the promoters/enhancers of both *SILV* and *MLANA*, and were found to be occupied by endogenous MITF using chromatin immunoprecipitation. Reporter assays, quantitative-PCR, Northern, and Western analysis all suggested that MITF either regulates reporters or the endogenous genes within melanoma cells and melanocytes. Protein and mRNA levels of the three antigens were found to correlate in a panel of melanoma cell culture lines as well as human primary melanocytes. Finally, we show MITF, *SILV* and *MLANA* mRNA levels are highly correlated in a primary human melanoma gene expression data set. Together, the results suggest that MITF transcriptionally regulates

*SILV* and *MLANA*, placing three commonly used diagnostic markers within a common transcriptional pathway.

## Materials and Methods

### Cell Culture and Media

501mel was a gift from Dr. Ruth Halaban (Yale University School of Medicine) and maintained in Ham's F10 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) or RPMI (Mediatech) with 10% FBS. SKMEL5 and A375 melanoma cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). MALME-3M, SKMEL2, SKMEL28, UACC62, UACC257 and M14 melanoma lines were all obtained from NIH (Frederick, MD). All melanoma cell lines were maintained in RPMI with 10% FBS. 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech) with 10% FBS. Human primary melanocytes were isolated as described previously<sup>56</sup> and maintained in TICVA medium (F10 with penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA), 7.5% FBS, 50 ng/ml TPA (Sigma), 225  $\mu$ mol/L IBMX (Sigma), 1  $\mu$ mol/L  $\text{Na}_3\text{VO}_4$  (Sigma), and 1 mmol/L dbcAMP; Sigma).

### Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays (ChIPs) were performed with cells grown in logarithmic phase. Cells were harvested by scraping and homogenized in a hypotonic buffer (10 mmol/L Tris-HCl, pH 7.4, 15 mmol/L NaCl, 60 mmol/L KCl, 1 mmol/L EDTA, 0.1% Nonidet P40, 5% sucrose, and 1X Complete proteinase inhibitor cocktail (Roche, Indianapolis, IN)) on ice using a Dounce homogenizer. The nuclei were isolated by centrifugation onto a 10% sucrose pad and then cross-linked with 1% formaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature with gentle shaking. Nuclei were then spun down and resuspended in ChIPs buffer (10 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 60 mmol/L KCl, 0.1% Nonidet P40, and 1X Complete proteinase inhibitor cocktail (Roche)) and sonicated by two 1-minute pulses on ice using a Fisher dismembrator fitted with a microtip. Rabbit antibodies against human MITF, anti-GST (Roche), and anti-Acetylated-Histone3 (Upstate, Waltham, MA) were then added to a 10-fold ChIPs buffer diluted sample and incubated on a nutator for 3 hours at room temperature. Ultralink protein-A/G-beads (Pierce, New York, NY) were added to the sample and a control sample and incubated for an additional hour. Immunoprecipitates were then washed twice with ChIPs buffer, twice with 500 mmol/L NaCl ChIPs buffer, and once with TE, pH 8.0. The immunoprecipitates were released from the beads by incubating at 65°C for 20 minutes in 1%SDS/TE and protein digested by proteinase K treatment side-by-side with an additional unprecipitated sample as input control. Cross-links were released by heating at 70°C for 10 hours and DNA recovered by extraction with phenol and chloroform at high salt (0.6 mol/L Na acetate, pH 8.0) followed by ethanol precipita-



tion. Semi-quantitative PCR was then performed on samples to amplify a fragment containing the intronic E-box (E1) or the upstream E-box (E2) in the human SILV transcription initiation region. The forward and reverse primers for the E1 region are 5'-CAT AAG ATA CCC CAT TCT TTC TCC ACT T-3' and 5'-GAG AAT GTG GTA TTG GGT AAG AAC AC-3', respectively. The primers for the E2 region are 5'-CAT GGA GAA CTT CCA AAA GGT GG-3' and 5'-TAC TCT CCC CAG GGA GTA TAA GT-3'. The MLANA promoter and control regions were analyzed similarly. The primers for MLANA promoter region are: 5'-TTG GAA TAA ATT GGG CTA CGA ACT T and 5'-TGG CAG GAT CTC AGC TCA CTA CAA C. The primers for MLANA downstream control regions are: 5'-ATG CCT GGC CTC TAT CCA CT and 5'-GGC GAC AGA GTG AGT GAG AT.

### EMSA (Electrophoretic Mobility Shift Assay)

Nuclear extracts were prepared as previously described.<sup>57</sup> Probe/competitor double-stranded oligonucleotides were 30 bp and spanned the individual E-boxes. A MLANA-specific double-stranded probe spanning the E2 site was prepared using the following sequences for sense oligos: wild-type probe, 5'-TTTCCATGTTACGGTGTGAGATATGC; mutant probe, 5'-TTTCCATGTTGAGGTGTGAGATATG. The SILV probe was prepared using the following sequences for sense oligos: wild-type probe, 5'-CCCAGAGCCCTTTCATGTGATGCTCAGCT; mutant probe, 5'-CCCAGAGCCCTTGTAGGTGATGCTCAGCT. Probe labeling was performed using T4 polynucleotide kinase (New England BioLabs, Beverly, MA) and  $\alpha$ -[<sup>32</sup>P]-ATP (PerkinElmer, Boston, MA) to label the 5' oligo before annealing. Binding reactions contained 25 fmol (~100,000 cpm)-labeled probe, 1  $\mu$ g poly (dI-dC) (Amersham Biosciences, Piscataway, NJ), 5% glycerol, 0.1 mol/L KCl, 10 mmol/L Tris (pH 7.6), 0.2 mmol/L DTT, 1  $\mu$ l (5 mg/ml) human 501 melanoma nuclear extract, and 4 to 8  $\mu$ l D5 anti-human Mitf mAb (Neomarkers, Fremont, CA) in a 20  $\mu$ l reaction volume. Reactions were incubated for 30 minutes at room temperature and loaded on 6% 0.5X TBE non-denaturing gels. For competition studies, indicated amounts of molar excess double-stranded mutant probes were included in the binding reactions.

### Construction of SILV and MLANA Reporters

A 2.8-kb fragment containing the first exon of SILV and flanking sequences was amplified from human genomic DNA (Roche) using primers 5'-GCA ATC CTA TGA CCA CGG CCT CCC AAA G-3' and 5'-GTT TTC CAT GGA TTC CAC ACC ACC CTA TAC-3'. A smaller fragment was subsequently amplified from the 2.8-kb fragment with primers 5'-CCG GTA CCC ACA TAA CTC CAC TCC ATG GAG-3' and 5'-CCC CAT GGC CAG CTA ATT TTT GTA CTT TTA-3', digested with *Kpn*I and *Nco*I, and inserted into pGL3-basic vector (Promega, Madison, WI). Subsequently, the splice acceptor of SILV exon 4 was amplified with primers 5'-ttc CAT Ggc ctc aac ccc caa cta ml-3' and 5'-ccC CAT GGg gct ccc tga aag ata a-3' and

inserted into the *Nco*I site. The initiation ATG of luciferase was cloned into the comparable location of the MLANA gene to generate a luciferase reporter including 1.9 kb of the MLANA promoter upstream of the transcriptional start site. MLANA promoter was amplified with primers: 5'-ACT GTT TGG TGG TCT CTG CTG GTC TGA T and 5'-GCT GGC TGG CCG CGT GTA TGA, and inserted into the pCR4-TOPO vector (Invitrogen). The vector was then digested with *Eco*RI, the promoter fragment was filled in with Klenow, and inserted into pGL3-basic digested with *Sma*I.

### SILV and MLANA Reporter Construct Mutagenesis

Site-directed mutagenesis was performed using the Quick-Change-method (Stratagene, La Jolla, CA) according to the supplier's recommendations. Mutagenesis primers were designed as follows (using the following sense strands with their corresponding complementary oligonucleotides):

SILV-E1: 5'-CCCAGAGCCCTTGTAGGTGATGCTCAGCT-3'

SILV-E2: 5'-AAATCCGCCTGGAGAGGTGAGTGGCCTCT-3'

MLANA-E1: 5'-CAGCACCTAACCACCTCTCACACAACC-AATG-3'

MLANA-E2: 5'-GTTTCCATGTTACCTCTGAGATATGCC-TCC-3'

### Transfection and Dual-Luciferase Reporter Assay

For all reporter assays triplicate transfections of 293 cells were carried out in 24-well plates using Fugene-6 (Roche) according to manufacturer's recommendations. SILV reporter constructs were transfected together with either pRC-CMV/MITF (wt) (encoding full-length M-form human MITF)<sup>57-59</sup> or pcDNA3.1 (Invitrogen) and pRL-null (Promega). A total of 1  $\mu$ g DNA was used for each transfection. Cell lysates were prepared 48 hours after transfection using 100  $\mu$ l of passive lysis buffer as per manufacturer's recommendations (Promega). The MLANA promoter-luciferase fragment was cut from pGL3-Basic backbone with *Kpn*I and *Bam*HI, column-purified, and transfected with either pcDNA3/MITF (wt) (encoding full-length M-form human MITF) or pcDNA3.1 (Invitrogen) and pRL-TK (Promega). A total of 0.5  $\mu$ g of DNA was used for each transfection. Cell lysates were prepared 20 hours after transfection using 100  $\mu$ l of passive lysis buffer as per manufacturer's recommendations (Promega). All lysates were incubated for 15 minutes at room temperature. An aliquot was used to perform luciferase assays using the Dual-Luciferase kit (Promega) according to the manufacturer's recommendations, and signals were normalized for transfection efficiency by the internal Renilla controls.



### Adenovirus Infection and RNA Preparation

Adenoviruses encoding wild-type MITF, dominant-negative mutant MITF, or vector control encoding a fusion of GFP-*wee1* for nuclear localization, were generated as previously described.<sup>57</sup> Briefly, 10<sup>6</sup> human primary melanocytes were plated per 100-mm plate. On the second day, cells were overlaid with 2 ml of serum-free F10 media containing 10 mmol/L MgCl<sub>2</sub> and concentrated adenovirus was added at MOI 300. The cells were incubated at 37°C for one-half hour after which virus was removed and fresh full media was added. Total RNA was isolated with RNAqueous-4PCR kit (Ambion, Austin, TX) at 68 and 80 hours after infection according to manufacturer's instructions. SKMEL5 melanoma cells were infected at MOI 100 and the RNA was harvested at 52, 68, and 80 hours post-infection. UACC62 cells were infected at MOI 200 and RNA was harvested at 72 hours post-infection.

### Real-Time/Quantitative PCR

The real-time PCR primers for human *SILV* were 5'-TCT GGG CTG AGC ATT GGG-3' and 5'-AGA CAG TCA CTT CCA TGG TGT GTG-3'. The probe for human *SILV* was 5'-6-FAM-CAGGCAGGGCAATGCTGGGC-TAMRA-3' (Applied Biosystems, Foster City, CA). The total volume of each reaction was 25  $\mu$ l including 12.5  $\mu$ l 2X Master Mix without UNG (uracil-N-glycosylase), 0.625  $\mu$ l MultiScribe Reverse Transcriptase and RNase inhibitor (Applied Biosystems), 0.5  $\mu$ l of each primer (10  $\mu$ mol/L stock), 0.25  $\mu$ l of the probe (5  $\mu$ mol/L stock) and 1  $\mu$ l of the template at 100 ng/ $\mu$ l. Reverse transcription proceeded at 48°C for 30 minutes. Then, 40 cycles of PCR reaction were carried out at 95°C for 15 seconds and at 60°C for 1 minute. Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with analysis using the integrated Sequence Detection System Software Version 1.7. Standard curves were generated for all primer sets to confirm linearity of signals over the experimentally measured ranges.

### Northern Analysis

Total RNA was isolated from human UACC-62 melanoma cells using the Trizol reagent (Invitrogen). Ten  $\mu$ g of total RNA was resolved on a 1% agarose gel and blotted onto Hybond-N membrane (Amersham Biosciences) and cross-linked in a GS Gene Linker (Bio-Rad, Hercules, CA). The MLANA probe was prepared by random priming of the full-length reverse transcription-PCR-derived CDS for human MLANA. A probe for human GAPDH was prepared by a restriction digestion of the full-length GAPDH cDNA, followed by random priming of the purified fragment.

### Immunoblots

Melanoma cells and adenovirus-infected primary melanocytes were collected and lysed in 2X lysis/loading

buffer (125 mmol/L Tris, pH 6.8, 4.6% SDS, 20% glycerol, and 0.04% pyronin Y), resolved by electrophoresis in 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Protran from Schleicher & Schuell, Keene, NH). All proteins were detected using chemiluminescence and antibodies to *SILV* (anti-PEP13h, a gift from Dr. Vincent Hearing), MLANA (NeoMarkers AB-2, Fremont, CA), MITF (C5), SOX10 (Active Motif, Carlsbad, CA), BRN2 (Santa Cruz Biotechnology, Santa Cruz, CA), CREB/ATF1 (25C10G; Santa Cruz Biotechnology), phospho-CREB/ATF1 (Cell Signaling Technologies, Beverly, MA), or  $\alpha$ -tubulin (Sigma).

### Preparation of Genomic DNA and Analysis of BRAF Status (V599E Mutation) in Melanoma Cell Lines

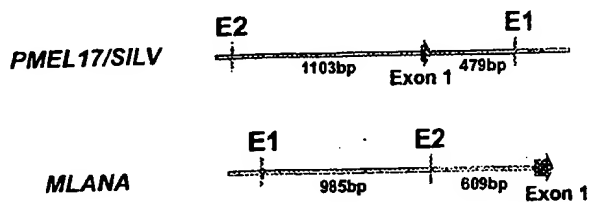
Genomic DNA from various melanoma cell lines was isolated with the DNeasy Tissue kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Fragments spanning the T1796A mutation were amplified with primers 5'-AAG CAT CTC ACC TCA TCC TAA CAC ATT T and 5'-CTT TCT AGT AAC TCA GCA GCA TCT CAG G from the genomic DNA. These fragments were subsequently sequenced with primer 5'-ATA GCC TCA ATT CTT ACC ATC CAC AAA to determine the mutation status.

### Microarray and Pearson Correlation Coefficient Analyses

Expression data on NCI 60 cell lines were collected as described elsewhere.<sup>60</sup> Expression profiling data on 190 human primary tumors were collected using Affymetrix Hu6800 high-density oligonucleotide microarrays as described elsewhere.<sup>61</sup> Expression values were given a lower threshold of 20 units and a ceiling of 16,000 units. The data set was filtered to eliminate genes whose expression levels did not vary more than fivefold or at least 100 absolute units across the data set. Of the 7129 genes on the microarray, 6466 genes passed this filter and were subjected to further analysis. The raw expression value for each gene was normalized to a mean of zero and SD of one across all samples in the data set. Degree of similarity analysis was performed using the Pearson correlation coefficient to identify genes with similar patterns of expression to MITF across the melanoma and non-melanoma collections, resulting in a sorting of the 6466 genes by their degree of correlation.

### Results

It has been previously shown that MITF modulates the transcription of several key enzymes in the pigment pathway and might regulate other melanocyte-specific differentiation-associated genes in melanocytes.<sup>62,63</sup> Of the melanocyte-specific melanoma-associated antigens, tyrosinase, TYRP1, and DCT are widely considered to be transcriptional targets of MITF.<sup>2,4,5</sup> Two remaining genes



**Figure 1.** The *SILV* and *MLANA* promoters/enhancers. Initial transcribed exons are denoted by large arrows and E-boxes are represented by vertical bars.

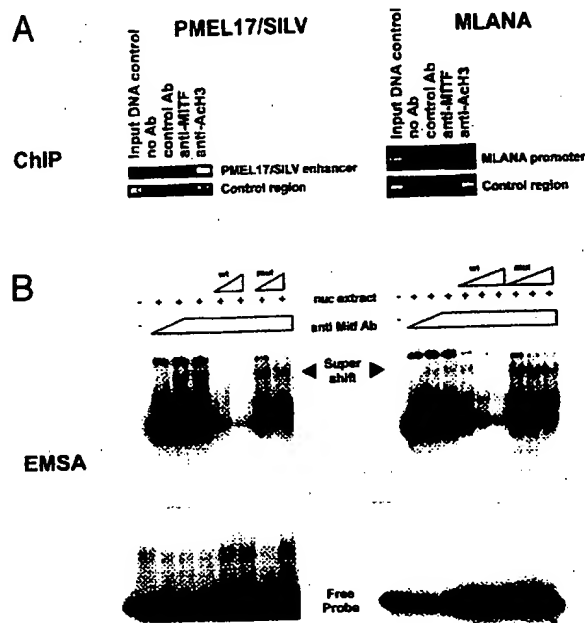
are *SILV* and *MLANA*, which encode proteins recognized by the widely used diagnostic antibodies, HMB45 and Melan-A, respectively. Based on the role of MITF in the regulation of melanocyte-specific genes, we asked whether MITF might also modulate the expression of these genes.

### Promoter/Enhancer Structures of *SILV* and *MLANA*

Since MITF modulates its targets through association with E-box elements in their promoters/enhancers (Figure 1), we examined human genome sequences surrounding *SILV* and *MLANA* transcriptional initiation sites. For *SILV*, one E-box (CACGTG) was found 1103 bp upstream its start and a second E-box (CATGTG) was identified in a region within the first intron. This intronic E-box contains a 5'-flanking T which was previously reported to contribute to the recognition of CATGTG by MITF.<sup>64</sup> For *MLANA*, two E-boxes were identified upstream of its transcriptional start site at -609 and -1594 bp, respectively. The distal site matches the consensus CACATG while the proximate site is a canonical (CACGTG) E-box that has been previously reported to be responsible for melanocyte specificity of the promoter, though the responsible transcriptional regulator was not known.<sup>65</sup> Murine homologues of *SILV* and *MLANA* were also examined, and were found to contain E-boxes in similar positions as the human genes (data not shown).

### In Vitro and in Vivo Binding of MITF to the *SILV* E1 Region and *MLANA* Promoter Region

To examine *in vivo* chromatin occupancy by MITF and help establish the directness of potential transcriptional regulation, chromatin immunoprecipitation (ChIP) was carried out from human melanoma cells (Figure 2). Primers were designed which span the upstream and intronic E-boxes located within the *SILV* transcription initiation region. Immunoprecipitation with antibody against MITF did reveal occupancy by MITF of the E1 enhancer region (*SILV* enhancer), but not a control region located near the E2 site (Figure 1) located further upstream. Additional specificity controls included no antibody or control polyclonal antibody (directed against GST protein). An antibody directed against acetylated histone H3 represented a positive control in each reaction. For *MLANA*, ChIP revealed occupancy by MITF of the E-box containing



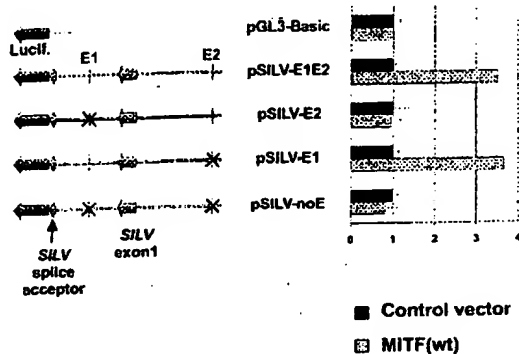
**Figure 2.** Binding of MITF to *SILV* enhancer and *MLANA* promoter *in vitro* and *in vivo*. **A:** Chromatin immunoprecipitations were performed on materials isolated from melanoma cells. DNA from lysates before immunoprecipitation was used as positive input control. The *SILV* enhancer primer set amplifies the intronic regulatory region which is important for gene expression, while the control primer set amplifies the region containing the upstream E-box which is dispensable for gene expression (negative control). The *MLANA* promoter primer set spanning the upstream E-boxes, while the control primer set amplifies a portion of the 3' UTR (negative control). **B:** EMSA assays were performed on melanoma lysates using probes containing *SILV* intronic E-box (E1) and *MLANA* proximate E-box (E2), respectively. Similar binding of MITF was seen at the E1 site of *MLANA* as well (data not shown).

promoter region. No occupancy was seen for MITF at the 3' UTR ("control region"), and additional controls included no antibody or comparable polyclonal anti-GST antibodies. Although ChIP does not permit accurate assessments of precise sequence elements being contacted by the transcription factor, it does suggest that MITF is bound to promoter/enhancer elements near the conserved E-boxes for both genes.

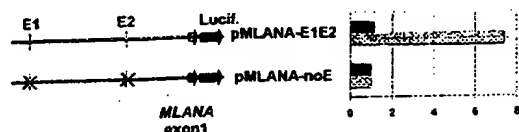
To specifically examine DNA binding by MITF of the relevant E-boxes, EMSA assays were performed using DNA probes containing these E-box elements plus melanoma nuclear extracts (as a source of MITF) and a monoclonal anti-MITF antibody (D5) for supershift analysis. Supershifted complexes were observed on addition of wild-type radiolabeled probes to the *SILV* E1 or *MLANA* E2 and E1 (data not shown) boxes in the presence of anti-MITF antibody (Figure 2). More importantly, the supershifted bands were specifically competed away with increasing titration of wild-type unlabeled probe, but not with identical titration of point mutated cold probes (Figure 2). Together, these results suggest that MITF directly binds the intronic E-box element in the *SILV* enhancer and the proximate E-box upstream of the *MLANA* transcriptional start site.

## Reporter Assays:

### PMEL17/SILV point mutants



### MLANA point mutants



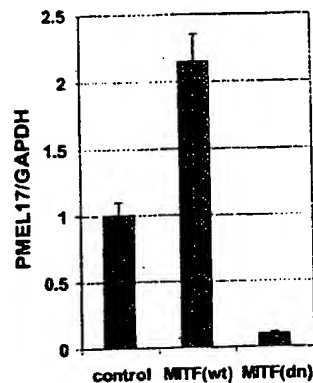
**Figure 3.** Reporter assays of SILV and MLANA point mutants. SILV and MLANA reporters were transfected into 293 cells. Firefly luciferase activities in samples were normalized to Renilla luciferase activities in the same specimens. Relative luciferase activities are shown. To recapitulate the putative SILV intronic enhancer, the genomic locus was incorporated into the pGL3 reporter with luciferase placed downstream of a SILV splice acceptor sequence. As described in Results, correct splicing of the reporter transcript was separately confirmed.

### E-Box-Dependent Modulation of SILV and MLANA Reporters by MTF

To further test the role of specific promoter/enhancer elements in the regulation of *SILV*, luciferase reporters were constructed which recapitulate the intronic location of the E-box element for its transcription. As shown in Figure 3, the intact genomic region was used as a template for the reporter. Transcription initiation should occur at the *SILV* exon 1 site, and the intron-containing transcript should be spliced to a downstream *SILV*-derived splice acceptor (which is fused to luciferase). To verify that the template was correctly initiated and spliced, RT-PCR was used together with 5' RACE of transfected cells. Sequencing of the resulting products confirmed that the luciferase reporter did correctly initiate and splice the luciferase transcript to exon 2 (data not shown). Using this reporter, wild-type MTF was found to stimulate luciferase activity (Figure 3, pSILV-E1E2), and point mutagenesis revealed an essential role for the intronic E-box element (E1) in mediating MTF-dependent transactivation. These data are consistent with the notion that the conserved E1 element may function as an intronic enhancer for MTF-regulated *SILV* expression.

*MLANA* promoter activity was also assessed using luciferase reporters. 1.9 kb of the *MLANA* gene upstream of its transcriptional start fused to the luciferase ATG was used in reporter assays. Wild-type MTF significantly up-regulated the wild-type reporter (pMLANA-E1E2) (Figure 3). Point mu-

## A. Quantitative RT-PCR for PMEL17/SILV



## B. Northern for MLANA



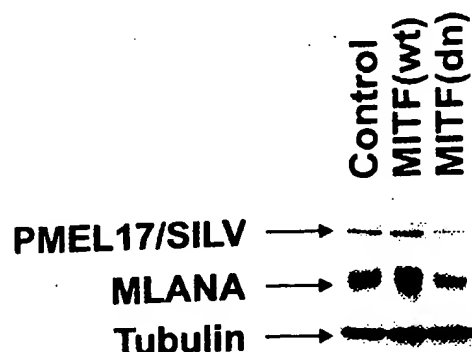
**Figure 4.** Regulation of endogenous *SILV* and *MLANA* mRNA levels by MTF. **A:** *SILV* mRNA levels were assayed after adenovirus infection (top panel). Total RNA was isolated from melanoma cells infected with adenoviruses 72 hours after infection. Quantitative RT-PCR was performed and data were normalized to endogenous GAPDH levels. Expression levels for MTF (wt) and MTF (dn)-virus infected samples were normalized to the control samples. **B:** *MLANA* mRNA levels were assayed after adenovirus infection (bottom panel). Total RNA was harvested 72 hours after adenovirus infection and Northern analysis was performed.

tations of both E-boxes completely abolished responsiveness to MTF (Figure 3). These data suggest that the modulation of *MLANA* expression by MTF is E-box-dependent and further supports the directness of this regulation.

### Transcriptional Regulation of Endogenous *SILV* and *MLANA* by MTF

To investigate whether MTF regulates endogenous *SILV* and *MLANA* mRNA levels in the melanocyte lineage, early passage human primary melanocytes and multiple human melanoma cell lines (501mel, SKMEL5, UACC-62 and A375) were infected with adenoviruses overexpressing either control, wild-type MTF or dominant-negative MTF (R218del). The R218 deletion mutant of MTF is derived from a dominantly inherited basic domain mutant mouse allele which preserves dimerization, but ablates DNA binding and thus sequesters wild-type dimerization partners in non-functional heterodimers. This allele has thus been ex-

## Western Analysis



**Figure 5.** Regulation of endogenous SILV and MLANA protein levels by MITF. Western analysis was performed on melanoma lysates harvested 72 hours after adenovirus infection.

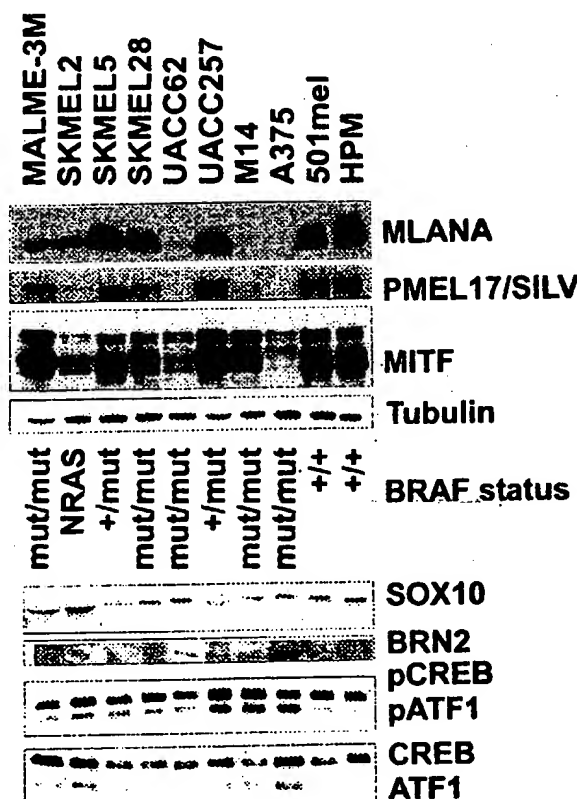
tensively used as a specific dominant-negative mutant.<sup>2,56,57</sup> Adenoviral infection efficiency approached 100% in these cells, based on GFP signal (data not shown) and was shown to produce strong expression of wild-type or mutant MITF by Western blot (data not shown). Enhanced or disrupted DNA binding by MITF in nuclear extracts occurred by about 48 hours post-infection, lasting until at least 96 hours post-infection, as measured by EMSA analyses (data not shown). Total RNA was isolated after 72 hours. Real-time (quantitative) PCR was performed for SILV on the RNA samples. Wild-type MITF significantly stimulated SILV mRNA expression while dominant-negative MITF suppressed SILV mRNA levels (Figure 4A). MLANA expression was examined by Northern blot analysis (due to unknown complications with quantitative PCR). Wild-type MITF up-regulated, while dominant-negative MITF suppressed MLANA mRNA levels (Figure 4B) relative to vector control adenovirus (normalized to GAPDH as a control).

Protein levels of SILV and MLANA were also examined following up- or down-regulation of endogenous MITF using adenoviruses, as above. Western blotting for SILV and MLANA (Figure 5) revealed increases of both in the presence of wild-type MITF and decreases of both in the presence of dominant-negative MITF. These data are consistent with the hypothesis that MITF regulates expression of SILV and MLANA in the melanocyte lineage.

### Comparison of MITF, SILV, and MLANA Expression in Cultured Melanomas and Melanocytes

Next, we examined protein levels of these genes in a panel of 11 human melanoma cell lines as well as primary early passage human melanocytes. As shown in Figure 6, a general pattern became apparent when comparing expression of MLANA, SILV, and MITF. A relatively clear pattern of correlated expression was seen for MITF, SILV, and MLANA in these specimens.

Overall, considerable variability was seen in MITF expression levels among the various cell sources. Yet, MITF



**Figure 6.** Correlation between MITF, MLANA, and SILV expression and BRAF mutation status. Western analysis were performed on cell lysates harvested from various melanoma cell lines and human primary melanomas with antibodies specified in Materials and Methods. BRAF mutation status was assessed by PCR and subsequent sequencing on corresponding genomic DNA. Whereas MITF and SILV expression correlated quite closely, no simple correlation was seen between MITF and BRAF mutational state or the various transcriptional regulators shown (which have been suggested to regulate MITF expression in specific contexts).

is clearly expressed in all of these samples, including A375, which contains significantly less visible protein by Western blot. To determine whether other potential upstream factors may similarly correlate with the pattern of MITF, SILV, and MLANA expression (and therefore be candidate regulators of MITF), we examined the expression of several transcription factors that have been previously demonstrated to modulate MITF expression. SOX10 binds multiple sites in the MITF promoter in melanocytes, and its mutation in humans is associated with a similar pigmentation/deafness phenotype as MITF deficiency.<sup>9,66-68</sup> The BRN2 transcription factor has been suggested to regulate or interact with MITF.<sup>69,70</sup> CREB/ATF1 have been shown to modulate MITF expression in melanocytes through recognition of a consensus CRE element, in response to cAMP signaling which is downstream of the melanocyte stimulating hormone.<sup>71-73</sup> As shown in the bottom panel of Figure 6, the expression levels of these factors did not correlate in a simple fashion with MITF or the other melanocytic markers.

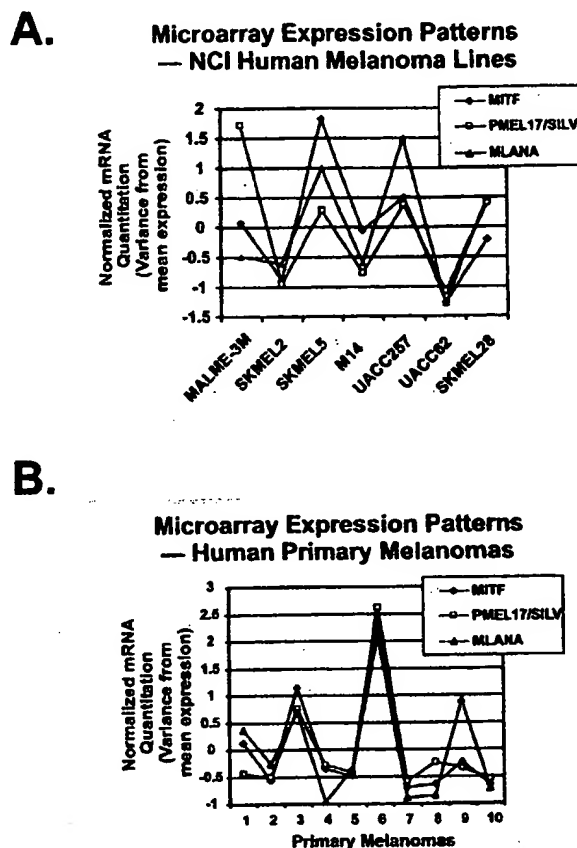
In addition, post-translational modifications of MITF have also been observed. One of these is mediated by the MAP-kinase pathway, which leads to the phosphorylation of

Ser73 in MITF by ERK1 and ERK2.<sup>56</sup> This post-translational modification leads to recruitment of the p300 coactivator and concomitant degradation of MITF protein.<sup>57</sup> Recently, it was found that oncogenic mutant BRAF(V599E) occurs in the majority of human melanomas studied.<sup>74</sup> Since BRAF activates the MAP kinase pathway, it might modulate MITF expression (either post-translationally or possibly even transcriptionally by unknown intermediates). This prompted us to analyze the mutational frequency of BRAF in the panel of melanomas and see whether this could explain the varied levels of MITF protein found (Figure 6, middle). A weak trend suggested that most abundant expression of MITF, MLANA, and SILV occurs in the setting of wild-type BRAF, but expression was otherwise variable and did not correlate clearly with BRAF status. Quantitative RNA measurements similarly demonstrated no simple correlation between BRAF mutational status and MITF mRNA levels (data not shown). Thus, no simple relationship emerged from these studies of other transcription factors and regulatory pathways that might explain the variable expression of MITF, SILV, and MLANA among melanomas.

#### Analysis of SILV, MLANA and MITF Expression in Tumor Microarrays

Several of the cell lines used in our analysis of protein levels are part of an NCI database on which transcriptional profiling has been performed.<sup>60</sup> Since protein expression patterns reflect both transcriptional and post-transcriptional regulation, we sought verification at the mRNA level that MITF, SILV, and MLANA expression also correlated. Indeed, it has been demonstrated that at least one mechanism which likely confers amelanotic (unpigmented) phenotype to certain melanomas is the post-translational degradation of pigmentation factors, specifically tyrosinase.<sup>15</sup> As shown in Figure 7A, the levels of MITF, SILV, and MLANA fluctuate together in a similar pattern among the cell lines available for analysis.

Finally, we wished to examine whether mRNA expression levels of MITF, SILV, and MLANA correlate within primary clinical melanoma specimens (rather than cultured cell lines which have been subjected to selective pressures of unknown clinical relevance). We therefore examined microarray expression profile data from a large unbiased data set of primary human cancers, including a series of 10 melanoma clinical specimens.<sup>61</sup> To find genes highly correlated with endogenous MITF expression in melanomas, we identified the genes best matching the MITF expression pattern using a Pearson correlation coefficient ranking of similarity.<sup>75</sup> Of the 6466 genes analyzed, both *SILV* and *MLANA* were among the top 20 genes correlating with levels of MITF in the primary melanomas of this series (Figure 7). Importantly, neither gene was significantly correlated with MITF expression in non-melanoma tumors due to lack of expression of *SILV* and *MLANA* (data not shown). Although this correlation does not prove a functional relationship between these factors, it utilizes a human primary tumor data set to help validate the model that MITF transcriptionally regulates the *SILV* and *MLANA* genes.



**Figure 7.** Correlations between MITF, SILV, and MLANA mRNA levels in cultured melanoma cell lines and human primary melanomas. NCI human melanoma cell lines (A) and human primary melanomas (B). Quantitative mRNA levels for all three genes were acquired from Affymetrix microarrays as described in Materials and Methods, and deviations from mean expression are plotted across each series.

#### Discussion

The results of the present work demonstrate that the essential melanocyte-specific transcription factor MITF regulates expression of the genes encoding the melanoma tumor markers MLANA and SILV. Evidence in support of this model includes the presence of conserved MITF consensus DNA binding elements within enhancer/promoter elements of both genes that are shown to be bound by endogenous MITF protein in melanoma cells. Furthermore MITF regulates luciferase reporters for these genes in a manner dependent on those DNA elements. MITF up- or down-regulation is seen to correspondingly modulate expression of MLANA and SILV in parallel directions, at both mRNA and protein levels. Across several melanoma cell lines, MITF, SILV, and MLANA correlate in expression at the protein level. Finally, expression microarray data reveal that MITF, MLANA, and SILV behave similarly in cultured cell lines as well as in primary melanoma specimens.

The *MITF* gene contains multiple alternative promoters which are capable of contributing unique initial coding exons to the common body of the gene. One of the alternative promoters is melanocyte-specific and subject

to MSH/cAMP regulation.<sup>71-73</sup> However, isoforms expressed from alternative promoters are present in other tissues, including osteoclasts and mast cells. As melanocyte-restricted target genes, MLANA and SILV will likely provide an opportunity to study whether their melanocyte restricted expression arises through unique activity of the melanocyte isoform of MITF or whether other transcription factors might contribute (together with MITF) to confer melanocyte-specific expression.

### *Levels of the Three Major Melanoma Markers Are Tightly Correlated with Each Other*

Despite the generally correlated protein expression levels for MLANA, SILV, MITF, no clear transcriptional regulator upstream of MITF expression could be identified on the basis of matching expression levels. For certain transcription factors, key post-translational events (such as phosphorylation of CREB) may modulate activity more than expression levels. Another important possibility is that MITF is transcriptionally regulated in a combinatorial fashion by multiple upstream regulators, whose correlations with MITF expression would thus appear complex. This model is particularly attractive as a means of understanding how ubiquitous factors, such as CREB, can regulate tissue-restricted target genes. Through cooperative linkages with other upstream regulators at the MITF promoter, such combinatorial networks might produce highly restricted target gene expression, using relatively ubiquitous upstream factors.

We are also interested in exceptions to the simple correlations in MITF, SILV, and MLANA protein expression patterns, and have found a single melanoma cell line (not among the NCI cancer cell data set) for which MLANA expression appears under-represented relative to MITF and SILV (data not shown). Multiple models could account for this discrepancy, including the possibility that transcriptional regulators other than MITF may also be rate-limiting in specific contexts. Alternatively post-translational differences could account for such discrepancies. For example, Halaban and colleagues<sup>15</sup> have previously examined the mechanism underlying lack of pigment production in amelanotic melanomas, and discovered aberrant tyrosinase protein degradation. It is plausible that components of the pigmentation machinery as well as byproducts of pigmentation chemistry may be toxic to melanoma cells and therefore a selective pressure may exist to disrupt components of the pathway in selected tumors. Of note, previous studies have demonstrated that oncogenic transformation associated with dysregulation of E2F may be connected to down-regulation of MITF.<sup>76</sup> In fact, these studies led to the previous suggestion that MITF may transcriptionally regulate the SILV gene, as is mechanistically demonstrated here.

### *Melanoma Markers and Prognosis*

It has been observed that low levels of MLANA and MITF correlate with a worse prognosis.<sup>17,37,53-55</sup> It is likely that in many of these tumors there is not a selective down-

regulation of a single antigen, but rather a coordinated decrease of many target genes, governed largely by MITF and its central regulation of pigmentation as the most observable of differentiation pathways. Thus, higher levels of antigen expression represent a more differentiated state, while lower levels represent a less differentiated state. Such regulation could be of potential therapeutic value if MITF expression were modulated.

### *Modulating Antigen Expression via MITF*

Notably absent from the literature are cases of melanoma antigen up-regulation in metastatic disease. The trend appears to be toward an expected state of a less differentiated phenotype with loss of many markers of differentiation. While previous work has previously linked MITF to the regulation of pigment genes and work presented here shows MITF regulation of SILV and MLANA, we have shown in an earlier study that MITF transcriptionally modulates the apoptosis modulator, Bcl-2. In addition, suppression of MITF through a dominant-negative mutant triggers apoptosis in melanoma cells while overexpression of Bcl-2 at least partially rescues this effect.<sup>56</sup> Bcl-2 expression also correlates closely with MITF in primary melanoma expression profiles.<sup>56</sup> Therefore, the worsened prognosis which has been suggested for melanomas expressing lower levels of MITF, occurs despite lower expression of the anti-apoptotic Bcl-2 factor. One model to explain this observation is that lower levels of MITF expression might select for tumor cells that are less differentiated (rather than expressing higher levels of survival genes). Additional studies will be needed to examine this question. Even though MITF expression fluctuates among melanomas, some level of staining is retained in nearly all melanomas and has been demonstrated to be a specific and sensitive diagnostic marker. Together, these observations underlie MITF's apparent dual roles in regulating both survival and differentiation in the melanocyte lineage.

The cytokine  $\alpha$ -MSH up-regulates MITF expression via a well-studied signal transduction pathway that also leads to increased pigment enzyme expression.<sup>71-73</sup> This pathway would therefore be expected to up-regulate MLANA and SILV expression as well. The combination of  $\alpha$ -MSH treatment with immunotherapy directed against antigens such as MLANA and SILV could potentially lead to therapeutic benefit both by increasing tumor cell differentiation and increasing the expression of melanoma-associated antigens that have been associated with therapeutically meaningful responses.

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